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Impact of different standard red blood cell storage temperatures on human and canine RBC hemolysis and chromium survival

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Abstract

BACKGROUND—Storage temperature is a critical factor for maintaining red-blood cell (RBC) viability, especially during prolonged cold storage. The target range of 1 to 6°C was established decades ago and may no longer be optimal for current blood-banking practices.

STUDY DESIGN AND METHODS—Human and canine RBCs were collected under standard conditions and stored in precision-controlled refrigerators at 2°C, 4°C, or 6°C.

RESULTS—During 42-day storage, human and canine RBCs showed progressive increases in supernatant non-transferrin-bound iron, cell-free hemoglobin, base deficit, and lactate levels that were overall greater at 6°C and 4°C than at 2°C. Animals transfused with 7-day-old RBCs had similar plasma cell-free hemoglobin and non-transferrin-bound iron levels at 1 to 72 hours for all three temperature conditions by chromium-51 recovery analysis. However, animals transfused with 35-day-old RBCs stored at higher temperatures developed plasma elevations in non-transferrin-bound iron and cell-free hemoglobin at 24 and 72 hours. Despite apparent impaired 35-day storage at 4°C and 6°C compared to 2°C, posttransfusion chromium-51 recovery at 24 hours was superior at higher temperatures. This finding was confounded by a preparation artifact related to an interaction between temperature and storage duration that leads to removal of fragile cells

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CONFLICT OF INTEREST

Dr. Gladwin is a co-inventor of pending patent applications and planned patents directed to the use of recombinant neuroglobin and heme-based molecules as antidotes for carbon monoxide poisoning, which were recently licensed by Globin Solutions, Inc. Dr. Gladwin is a shareholder, advisor, and director in Globin Solutions, Inc. Additionally, and unrelated to carbon monoxide poisoning, Dr. Gladwin and Dr. Kim Shapiro are co-inventors on patents directed to the use of nitrite salts in cardiovascular diseases, which have been licensed by United Therapeutics and Hope Pharmaceuticals, and Dr. Gladwin is a co-investigator in a research collaboration with Bayer Pharmaceuticals to evaluate riociguat as a treatment for patients with sickle cell disease. The remaining authors have disclosed no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

with repeated washing of the radiolabeled RBC test sample and renders the test sample unrepresentative of the stored unit.

CONCLUSIONS—RBCs stored at the lower bounds of the temperature range are less metabolically active and produce less anaerobic acidosis and hemolysis, leading to a more suitable transfusion product. The higher refrigeration temperatures are not optimal during extended RBC storage and may confound chromium viability studies.

Storage conditions affect the quality of allogeneic red blood cells (RBCs) and the clinical response to transfusion. Storage quality can vary by preservative solution, type of container and filter, refrigeration temperature, and duration. The age of stored RBCs has recently been a focus for researchers. In the United States, the Food and Drug Administration (FDA) allows a maximum shelf-life of 42 days when using modern anticoagulant-preservative solutions¹ at refrigeration temperatures of 1–6 °C,² although retrospective and experimental work has suggested greater adverse effects as transfused cells approach the 42-day expiration date.^{3–5} The increased hemolysis after prolonged storage and release of cell-free hemoglobin (CFH) and non-transferrin-bound iron (NTBI) may increase trans-fusion risks in subgroups such as patients with invasive bacterial infections.⁶ At present, prospective clinical trials have not demonstrated a clear survival benefit for patients who receive fresher blood, although trial arms have been limited to RBC storage for 7 to 10 days versus 18 to 22 days, and included few RBCs close to the 42-day limit.³ Evidence to date has been insufficient to change storage guidelines.^{3,5,7–9}

A potential confounder for studies of storage duration is the refrigeration temperature. Neither retrospective studies nor clinical trials have separately considered whether cold storage temperature might impact RBC viability, especially at the extremes of storage duration. When compared to other factors affecting RBC quality, the range of temperature variation allowed during storage, which has not fundamentally changed in more than 70 years,¹⁰ has received far less attention.

Because refrigeration is a key regulatory variable in RBC storage, administration, and transport, a reexamination of the effect of storage temperature with contemporary RBC storage practices is long overdue. Therefore, we undertook a detailed investigation of the effects of storage temperature on RBC viability, using ex vivo human and canine RBCs, and in vivo radiolabeled RBC transfusions into animals. We hypothesized that the lower bounds of the regulated storage temperature range would result in greater recovery of trans-fused RBCs and less hemolysis with release of potentially harmful by-products such as CFH and iron.

MATERIALS AND METHODS

Experimental design

This study was designed to examine the impact of varying the RBC storage temperature and duration within the recommended ranges used in the United States and Europe. Modern RBC anticoagulant-preservatives and state-of-the-art refrigeration techniques were used.

In the first set of ex vivo experiments, human and canine RBCs were randomly assigned to a specific storage temperature for 6 weeks. Storage bag supernatant was sampled weekly during this 6-week period to examine indices of RBC hemolysis (CFH and NTBI) and quality of the storage milieu (base deficit and lactate levels). A detailed description of these experimental procedures and blood storage techniques can be found below and in the online Appendix S1 Details on Blood Banking Techniques, Animal Care, Chromium Studies, Laboratory Analysis and Statistical Method.

To correlate the findings of the ex vivo studies in an in vivo experimental setting, we used a well-described model of canine RBC transfusion to examine the effect of varying RBC storage temperature on hemolysis and RBC viability after transfusion.¹¹ An animal model was selected for ethical reasons, as the study design required transfusion with a large volume of nontherapeutic, older RBCs and a radioisotope marker. In this set of experiments, animals were transfused 20 mL/kg of canine RBCs stored for either 7 or 35 days at one of three standard temperatures (2°C, 4°C, or 6°C). For 72 hours after transfusion, we serially measured plasma markers of hemolysis (transferrin-bound iron [TBI], NTBI, and CFH levels) that are also storage cytotoxins. In addition, RBC viability after transfusion was evaluated using standard chromium-51 (⁵¹Cr) RBC survival assay. These studies were done to correlate the findings of ex vivo, pretransfusion storage hemolysis in humans and animals with in vivo, posttransfusion RBC viability in animals.

In a final set of experiments, we examined whether storage at different temperatures within the currently recommended range alters the ⁵¹Cr viability test itself. These experiments are essential to confirm if the ⁵¹Cr labeling is an accurate measure of RBC viability when varying storage temperature.

Canine ex vivo RBC storage protocol

The effect of increasing storage temperature on hemolysis was determined for canine RBCs stored over 42 days. Twelve units of canine RBCs were stored for 42 days at a randomly assigned temperature condition: either 2°C, 4°C, 6°C, or a variable temperature that alternated from 2°C to 6°C every 12 hours. Every 7 days for 42 days, all units were sampled aseptically for complete blood count, electrolyte panel, CFH, NTBI, lactate, and blood gas.

Human ex vivo RBC storage protocol

Human leukoreduced RBC units were obtained from the Department of Transfusion Medicine, Clinical Center, National Institutes of Health, after collection and processing according to FDA regulations from four healthy volunteers on three separate donations. RBCs were not pooled for these studies. Donations were performed every 8 weeks, as per FDA regulations,¹² such that blood from all donors was studied at all temperatures in random order. RBCs were stored using the AS-3 preservative-anticoagulant solution (Nutricel, Haemonetics Corporation) for 42 days at one of three randomized storage temperatures (2°C, 4°C, or 6°C).

All units were sampled serially for laboratory analysis on the day of donation and every 7 days over the 42-day storage period. Samples were analyzed for complete blood count,

electrolyte panel, CFH, NTBI, lactate, and blood gas analysis. On Day 42, RBCs were sampled immediately after removal from the refrigerator, and then again after 2 and 4 hours of incubation on a rocker at room temperature. These two additional samplings simulate the clinical scenario where RBCs are transfused within the currently acceptable maximum range of 4 hours after removal from refrigeration.¹³

Animal transfusion procedure

To minimize antigenic variability between samples and because a single donor canine could not safely provide sufficient RBCs for the four animals studied each week simultaneously, a pooled sample from eight donors was prepared. All RBC storage bags were treated under identical conditions according to FDA regulations.^{2,12,14}

One day prior to transfusion (Day 6 or Day 34), RBC bags were sampled for complete blood count, electrolyte panel, CFH, TBI, NTBI, lactate, and blood gas. Additionally, microbiological culture was performed to detect any bacterial contamination. At 7 or 35 days of storage, 4 units (2 each from a different refrigerator, determined randomly) were removed, and 20 mL/kg (equivalent to 2–4 RBC units for a human) were transfused into each animal. Any remaining volume was discarded.

For additional description of the experimental procedures and techniques in these three sets of experiments and statistical methods, see Appendixes S1 and S2, available as supporting information in the online version of this paper.

Study approval

All experiments were conducted after protocol approval (#CCM-17-01) by the Animal Care and Use Committee of the Clinical Center at the National Institutes of Health.

RESULTS

RBC storage at higher temperatures raises supernatant NTBI levels

We first examined the effects of time and temperature on the supernatant of human and canine RBCs stored in bags. Human RBCs demonstrated progressive increases in supernatant NTBI over 42 days when stored at 4°C ($p = 0.0007$) and 6°C ($p < 0.0001$), but not when stored at 2°C ($p = 0.40$) (Fig. 1A). When compared to 2°C, RBCs stored at 6°C demonstrated a significantly greater rise in NTBI ($p = 0.008$), while a nonsignificant trend was observed at 4°C ($p = 0.09$). When maintained at room temperature for 4 hours, human RBCs demonstrated a similar ($p = 0.75$ comparing the three temperatures), progressive decrease in NTBI across all temperatures (combined, $p = 0.0007$) (Fig. 1B). The decrease was significant at 2°C and 6°C ($p = 0.05$ and $p = 0.01$) but showed a nonsignificant trend at 4°C ($p = 0.10$). During the 4 hours at room temperature, after accounting for the common slope, RBCs stored at 2°C exhibited significantly lower NTBI compared to 4°C ($p = 0.04$), and, although not significant, trended in the same direction at 6°C ($p = 0.08$).

Canine RBCs also exhibited increases in NTBI over 42 days at 2°C, 4°C, and 6°C (all, $p < 0.0001$). This rise in supernatant NTBI was significantly greater at 6°C versus 2°C ($p < 0.0001$), but not at 4°C versus 2°C ($p = 0.69$) (Fig. 1C). NTBI increased for canine RBCs

stored under the alternating 2°C and 6°C refrigeration regimen ($p < 0.0001$), which did not differ from the cells maintained continuously at 2°C ($p = 0.41$) (Fig. 1D).

RBC storage at higher temperatures raises supernatant CFH levels

Human RBCs experienced progressive increases in CFH over 42 days of storage at all storage temperatures (2°C: $p = 0.0004$; 4°C: $p < 0.0001$; 6°C: $p < 0.0001$) (Fig. 2A). There was a further progressive increase in CFH over 4 hours at room temperature, independent of previous storage temperature (all, $p < 0.0001$) (Fig. 2B). There were no significant differences in the increases over 42 days and over 4 hours at room temperature in CFH levels at the different storage temperatures in Fig. 2A or 2B, respectively.

Canine RBCs also demonstrated a progressive increase in CFH across all temperatures (all, $p < 0.0001$). The increase was greater at 6°C versus 2°C ($p < 0.0001$) and 4°C versus 2°C ($p = 0.03$) (Fig. 2C). For cells maintained at alternating 2°C and 6°C, CFH also increased significantly ($p < 0.0001$). The CFH rise for RBCs kept at alternating temperatures was significantly greater than for cells maintained strictly at 2°C ($p = 0.0001$) (Fig. 2D).

RBCs stored at higher temperatures have lower supernatant base excess and higher levels of lactate

Human RBCs exhibited a decrease in base excess over 42-day storage (all, $p < 0.0001$). The fall in base excess levels was significantly greater at 6°C versus 2°C ($p < 0.0001$) and at 4°C versus 2°C ($p = 0.009$) (Fig. 3A). Lactate levels also progressively increased over 42-day storage at all temperatures (all, $p < 0.0001$) (Fig. 3B). Although lactate levels were nominally increased at higher-temperature storage throughout the 42 days, there was no statistically significant rise in levels with higher temperatures.

Canine RBCs also underwent a progressive fall in base excess at 2°C, 4°C, and 6°C (all, $p < 0.0001$), which was greater at 6°C ($p < 0.0001$) and 4°C ($p = 0.03$) than 2°C (Fig. 3C). Progressively increased lactate levels were also seen at all temperatures (all $p < 0.0001$), with the increase again greater at 6°C than 2°C ($p = 0.002$) and 4°C than 2°C ($p = 0.02$) (Fig. 3D).

Canine NTBI and TBI levels exhibit two peaks after transfusion with 35-day-old RBCs stored at 4°C and 6°C

Studies were undertaken to correlate supernatant acidosis, hemolysis, lactate, NTBI, and CFH levels following storage with in vivo, posttransfusion values in animals. At 7 days of storage, serum NTBI was not significantly different for animals receiving RBCs stored at 2°C compared to 6°C at 0 to 72 hours after transfusion. NTBI was not significantly different from baseline at 24 or 48 hours for RBCs stored at either 2°C or 6°C. By 72 hours, NTBI levels were elevated compared to baseline in animals receiving RBCs stored at 6°C for 7 days ($p = 0.001$), but only a trend at 2°C ($p = 0.07$) (Fig. 4A).

At 35 days of storage, NTBI increased by 4 hours after transfusion of RBCs stored at all temperatures (all, $p < 0.0001$). NTBI was greater for 6°C versus 2°C ($p = 0.002$) and for 4°C versus 2°C ($p = 0.004$) (Fig. 4B). Animals receiving RBCs stored at 2°C demonstrated

elevated NTBI levels at 12 hours after transfusion ($p = 0.02$), which returned to baseline from 24 to 72 hours. RBCs stored at 4°C also showed an increase in NTBI levels at 12 hours ($p < 0.0001$), which returned to baseline from 24 to 48 hours. Unexpectedly, these animals experienced a late elevation in NTBI at 72 hours ($p < 0.0001$). NTBI was higher for RBCs stored at 4°C for multiple time points relative to RBCs stored at 2°C (at 4 hours, $p = 0.004$; at 12 hours, $p = 0.02$; and at 72 hours, $p = 0.0001$). Animals receiving RBCs stored at 6°C experienced normalization of NTBI at 12 hours (earlier than for 2°C and 4°C) but also experienced the late elevation at 72 hours ($p = 0.0003$).

TBI should increase until saturation alongside increases in NTBI. Mean TBI levels increased at 4 hours compared to baseline following transfusion of RBCs stored at 2°C ($p = 0.02$) and 6°C ($p < 0.0001$) for 7 days (Fig. 4C) associated with nonsignificant rises in mean NTBI levels at that same time point (Fig. 4A). At 35 days, the changes in TBI levels over 72 hours mirror the findings for mean NTBI levels (Fig. 4D). For 7-day-old and 35-day-old blood (with the exception of 6°C versus 2°C at 12 and 24 hours), there are no significant differences in mean TBI levels comparing storage of RBCs at 4°C or 6°C versus 2°C. The lack of significant decreases at most of the time points observed for TBI in Fig. 4C and 4D could be attributed to saturation of circulating transferrin with hemolysis-associated iron release at all temperatures.

CFH in animals peaks after transfusion with 35-dayold stored RBCs at 6° C

Animals receiving RBCs stored at 2°C for 7 days experienced an increase in CFH at both 48 and 72 hours after transfusion ($p = 0.01$ and $p = 0.002$, respectively). RBCs stored at 6°C exhibited increases in serum CFH at 1, 24, and 72 hours after transfusion ($p = 0.04$, $p = 0.02$, and $p = 0.007$, respectively) (Fig. 4E). CFH did not differ significantly between animals transfused RBCs stored at 6°C versus 2°C at any time point.

Animals receiving RBCs stored at 2°C for 35 days experienced elevated CFH at 12 hours ($p = 0.001$) and 24 hours ($p = 0.001$) after transfusion compared to baseline, but not at 1 to 4 hours and 48 to 72 hours (Fig. 4F). Animals receiving RBCs stored at 4°C had significantly elevated plasma CFH at all time points from 4 to 72 hours (at 4 hours, $p = 0.009$; at 12 hours, $p = 0.0001$; at 24 hours, $p = 0.003$; at 48 hours, $p = 0.04$; and at 72 hours, $p = 0.01$). Animals transfused RBCs stored at 6°C had higher CFH at 1, 4, 12, and 24 hours after transfusion (all, $p < 0.001$), but no changes from baseline at 48 to 72 hours. CFH was not significantly different for 6°C versus 2°C or for 4°C versus 2°C at any time point.

Unexpected greater ⁵¹Cr recovery after transfusion of RBCs stored at 4°C and 6°C for 35 days

To correlate the ex vivo storage temperature abnormalities seen in storage bag supernatant with RBC viability in vivo, standard ⁵¹Cr RBC survival assays were performed in animals. After 7 days of storage, no significant difference in ⁵¹Cr recovery was observed for animals transfused RBCs stored at 6°C and 2°C (Fig. 5A). At 72 hours after transfusion, there was a substantial (though not statistically significant) decrease in ⁵¹Cr recovery after transfusion with radiolabeled RBCs stored at 6°C ($p = 0.07$) (Fig. 5A). From 24 to 72 hours after

transfusion, RBCs stored at 6°C showed a progressive significant decrease in ^{51}Cr recovery ($p = 0.03$), but no significant trend was seen for RBCs stored at 2°C.

At 35 days of storage, animals transfused with labeled RBCs stored at 2°C showed unexpectedly lower ^{51}Cr activity than animals receiving RBCs stored at 4°C and 6°C, at 24, 48, and 72 hours after transfusion (Fig. 5B). There was a progressive decrease in ^{51}Cr activity from 24 to 72 hours for RBCs stored at 4°C and 6°C ($p = 0.001$ and $p = 0.003$, respectively), but no significant trend was seen for RBCs stored at 2°C.

Fewer RBCs stored 35 days at 4°C and 6°C survived the ^{51}Cr labeling process

In a final set of experiments, we investigated the unexpected, paradoxical superiority of ^{51}Cr -RBC survival after transfusion of RBCs stored at higher temperatures for 35 days. We examined whether storage at different temperatures within the currently recommended range alters the ^{51}Cr viability test itself. We first compared the supernatant contents of the stored RBCs at different temperatures to gauge the 35-day storage of the ^{51}Cr test sample. We then determined prior to transfusion the effect on RBC count and hemoglobin concentration of washing, labeling, and concentrating on the ^{51}Cr test samples from RBCs stored at different temperatures for 35 days. The mean values of the supernatant pH over the 35-day storage fell significantly more for RBCs stored at 6°C and 4°C versus 2°C (Fig. 6, Panel IA). pH was substituted for base excess as a measure of acid-base balance because the base excess values shown elsewhere in the study were too low to be recorded at 6°C. The mean values after the 35-day storage of the supernatant lactate, NTBI and TBI (Fig. 6, Panel IB, IC, and ID) rose significantly more for RBCs stored at 6°C and 4°C versus 2°C. The mean values of the supernatant CFH (Fig. 6, Panel IE) were not significantly different for RBCs stored at 6°C and 4°C versus 2°C, over the 35-day storage.

Pooled canine RBCs demonstrated equivalent baseline RBC counts and hemoglobin levels after distribution into separate storage bags. When washed twice for ^{51}Cr labeling after 35 days of storage at 2°C, 4°C, or 6°C, mean RBC counts and hemoglobin levels were lower for RBCs stored at 6°C than at 2°C ($p = 0.01$, 0.005, respectively) (Fig. 6, Panel IIA and IIB).

DISCUSSION

During the past 50 years, few of the seminal studies advancing RBC storage practices have addressed how modern practices affect the optimal storage temperature.^{15–24} Storage temperature was examined in the late 1970s²⁵ during the development of modern preservative solutions. Similar to our findings, these studies showed that raising storage temperature from 2.5° to 5.5°C with modern storage solutions over 6 weeks increased lactate production and lowered pH. Some studies concluded that “temperature should be recognized as a potentially highly significant variable,”²² though others considered these differences small and of no practical importance.²⁵

The present study examines human and canine RBCs stored under modern conditions in precision-controlled refrigerators to maintain exact temperatures. We selected for study three temperatures at the bounds and midway within this range: 2°C, 4°C, and 6°C. At the higher

storage temperatures of 4°C and 6°C, progressive elevations in NTBI and CFH were observed in the storage bag over time. Both human and animal RBCs stored at 2°C overall showed smaller or no increase in NTBI and CFH under the same conditions. During storage, decreases in base excess and increases in lactate levels were also observed at the higher temperatures. It is likely that increased RBC metabolic function at higher temperatures resulted in more acid accumulation in the storage container and poorer storage conditions, which in turn resulted in greater hemolysis. We also observed a paradoxically lower recovery of chromium-labeled RBCs following transfusion of RBCs stored at lower temperatures, which may be due to a survival bias caused when prolonged storage at higher temperatures selects against “weaker” RBCs.

While small changes associated with increased metabolic activity can be seen after even short durations of storage, the effect requires weeks to develop a clinically detectable difference. In our animal model of transfusion, RBCs stored for just 7 days showed no difference in ^{51}Cr survival at 24 hours after transfusion, regardless of temperature. Although 24 hours is defined as the interval used for licensure, we observed at 48 and 72 hours that RBCs stored at 6°C showed progressively declining chromium survival not seen with RBCs stored at 2°C. This same phenomenon of delayed hemolysis over time was also seen after 35 days at higher cold-storage temperatures. The clinical consequences of delayed hemolysis with release of CFH and NTBI are unknown, but increased risks by promoting bacterial growth during infection or through nitric oxide scavenging worsening coronary syndromes, respectively, have been proposed.^{6,11,26}

In contrast to 7-day-old RBCs, RBCs transfused after 35 days at 4°C and 6°C showed superior survival at 24, 48, and 72 hours when compared to RBCs stored at 2°C. The apparently improved recovery occurred despite the increased hemolysis in the storage bags after 35 days and the higher NTBI and CHF for 72 hours after transfusion. We attribute the discrepancy to a storage-induced artifact affecting the ^{51}Cr assay. We reported previously that washing RBCs after prolonged storage could result in hemolysis of the more vulnerable cells that would be sufficient to alter transfusion study outcomes.²⁷ The ^{51}Cr labeling process includes two washing steps and a room temperature incubation interval. Cells washed after 35-day storage at higher temperatures showed reduced total cell counts. We suspect that metabolic injury at higher-temperature cold storage preferentially injures the “weaker” cells in the storage bag. These cells would be more susceptible to lysis during the washing steps. In that case, the cells that survive ^{51}Cr labeling represent a “stronger” cohort, not representative of the original cells in the container at the end of storage prior to transfusion. Supporting this hypothesis is the finding that more total cells remained after the labeling process in cells stored at 2°C.

These findings suggest that storage temperature may represent a previously unrecognized confounder for the standard chromium survival assay. The manipulation required of RBCs to label the sample with chromium under certain storage conditions can change the characteristics of the injected sample so that it is no longer representative of the RBCs that are being transfused. Only at 2°C with prolonged storage did we find chromium-labeled RBCs stable over days as has been previously reported. To ensure that elevated storage temperature within the accepted range does not confound the ^{51}Cr -labeled sample,

additional sampling could be performed at 48 and 72 hours to see if values are stable or decreasing. If these findings are confirmed with ^{51}Cr studies of human RBCs, the interpretation of the radiolabeling assay for evaluating RBC storage quality will have to be reevaluated.

Our studies of human and canine RBCs appear to indicate an interaction between storage time and temperature within the regulatory storage standards. The upper extreme of temperature (4–6°C) showed increased NTBI and CFH, both of which are potentially harmful to transfusion recipients.^{6,9,11,26} After transfusion of these RBCs into animals, we observed two peaks in serum NTBI and CHF. The initial peak in the first 24 hours could be attributed to substances transfused from the supernatant and rapid hemolysis of damaged donor cells. After return to normal levels over 24 to 48 hours, a second peak at 72 hours probably relates to delayed hemolysis of donor RBCs with less severe storage injury. As the study terminated at 72 hours, the persistence of the elevated NTBI and CFH is unknown. The clinical significance of delayed donor cell hemolysis is also uncertain, although it may represent an important secondary endpoint to consider when evaluating storage conditions.

The effect of higher refrigeration temperature may be underestimated because the experimental paradigm did not consider additional factors thought to influence viability, such as the time in clinical practice that RBCs are kept not fully refrigerated prior to the initiation of transfusion. In addition, after collection many RBC units are kept at ambient temperatures for up to 24 hours before processing to obtain platelets, and then may be transported at 10°C from the collecting blood center and between hospitals for up to 24 hours. The use of blood warmers was likewise uninvestigated. These data suggest that storage temperature is particularly important for the survival of cells after protracted storage, noting that RBCs stored at alternating temperatures (between 2°C and 6°C every 12 hours over 42 days) were identical to RBCs stored strictly at 4°C.

Human and canine RBCs produced consistent storage temperature findings with one exception. Human RBCs showed no lowering of the of CFH elevations in the supernatant during prolonged storage at 2°C. In contrast, these cells had lower supernatant NTBI levels with prolonged storage at 2°C. Likewise, animal RBCs with prolonged storage at 2°C produced both lower CFH and NTBI levels in the supernatant and, after transfusion, lower plasma levels of both. We find no plausible mechanism by which prolonged storage at lower temperatures would have different effects on NTBI and CFH release for human RBCs but not for canine RBCs. We attribute this isolated CFH finding by exclusion to studying a small number (three) of human RBC units.

In conclusion, blood storage practice standards may not adequately account for an important interaction between storage time and temperatures that may confound chromium RBC viability testing and prove clinically relevant. At the upper of bounds of the refrigeration temperature range, lower RBC viability and increased hemolytic byproducts are seen at the end of the shelf life. Early and ongoing hemolysis within the storage bag and in vivo would result in shorter intervals between transfusions, the need for additional transfusions, and increased iron deposition for chronically transfused patients. The washing procedure for chromium labeling combined with higher temperature storage causes lysis of a cohort of

cells, resulting in a test sample not representative of RBCs being transfused. Storage at refrigeration temperatures closer to 2°C may result in a better product for transfusion and more accurate for chromium RBC viability testing. Further studies are needed to determine the reproducibility of our findings in human volunteers and the magnitude of their clinical importance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CFH	cell-free hemoglobin
FDA	Food and Drug Administration
NTBI	non-transferrin-bound iron
TBI	transferrin-bound iron

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Fig. 1.

Serial supernatant NTBI levels for canine and human RBCs stored at over 42 days. The serial NTBI levels at different storage temperatures are shown by different geometric symbols. Each symbol represents an individual RBC unit. (A) Supernatant NTBI levels for human RBCs over 6 weeks of storage. (B) Supernatant NTBI levels for human RBCs after 6 weeks of storage that were then placed at room temperature for 4 hours but not transfused. (C) Supernatant NTBI levels for canine RBCs over 6 weeks of storage. (D) Serial supernatant NTBI levels for canine RBCs over 6 weeks of storage at alternating temperatures. The gray area in each panel is the normal range for NTBI levels based on data provided by the commercial group performing the assay (aFerrix Ltd., Tel Aviv, Israel). Individual data points were fitted for each storage condition to linear regression lines. The slopes were tested against the null hypothesis of 0 (see keys above), and then each slope was compared to that of 2° C. In B, we postulate the small falls in supernatant NTBI levels over 4 hours is due to changes in binding of iron to transferrin as temperature markedly rose from refrigeration to room temperature. NTBI = non-transferrin-bound iron.

Fig. 2.

Supernatant CFH levels for canine and human RBCs stored at different temperatures over 42 days. The format is similar to Fig. 1 except that serial CFH levels shown at different storage temperatures. (A) Supernatant CFH levels for human RBCs over 6 weeks of storage. (B) Serial supernatant CFH levels for human RBCs after 6 weeks of storage and being placed at room temperature for 4 hours (but not transfused). (C) Supernatant CFH levels for canine RBCs over 6 weeks of storage. (D) CFH for supernatant canine RBCs with alternating temperatures over 6 weeks of storage. The three gray lines in D are reproduced from the three regression lines in C. A logarithmic transformation was performed for CFH to normalize the data for analysis. CFH = cell-free hemoglobin.

Fig. 3.

Supernatant base excess and lactate levels for canine and human RBCs stored over 42 days. The format is similar to Fig. 1A and 1C, with serial base excess and lactate levels instead of NTBI levels at various storage temperatures. (A) Serial supernatant base excess levels for human RBCs over 6 weeks of storage. (B) Serial supernatant lactate levels for human RBCs over 6 weeks of storage. (C) Serial supernatant base excess levels for canine RBCs over 6 weeks of storage. (D) Serial supernatant lactate levels for canine RBCs over 6 weeks of storage. NTBI = non-transferrin-bound iron.

Fig. 4.

Plasma NTBI, TBI and CFH levels for canines transfused with RBCs stored over 7 and 35 days. Serial mean ([notdef] SE) plasma NTBI, TBI, and CFH for 72 hours after transfusion of canine RBCs shown. The serial mean NTBI, TBI, and CFH levels at various storage temperatures are shown by geometric symbols. (A) Serial mean plasma NTBI levels for RBCs transfused after 1 week of storage. (B) Serial mean plasma NTBI levels for RBCs transfused after 5 weeks storage. (C) Serial mean plasma TBI levels for RBCs transfused after 1 week of storage. (D) Serial mean plasma TBI levels for RBCs transfused after 5 weeks storage. (E) Serial mean plasma CFH levels for RBCs transfused after 1 week of storage. (F) Serial mean plasma CFH levels for RBCs transfused after 5 weeks of storage. The gray area in A and B represents the normal range for NTBI. A logarithmic transform for

CFH was performed to normalize the data for analysis. CFH = cell-free hemoglobin; NTBI = non-transferrin-bound iron; TBI = transferrin-bound iron.

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Fig. 5.

⁵¹Cr recovery for canines transfused with RBCs stored over 7 and 35 days. The percent recovery of canine RBCs using ⁵¹Cr-labeling testing at 24, 48, and 72 hours after transfusion. RBCs stored at different temperatures are shown by different shades of black and gray vertical rectangular bars. (A) Percent RBC recovery for transfusion after 7 days of storage at different temperatures. (B) Percent RBC recovery for transfusion after 35 days of storage at different temperatures. The dashed slanted lines in A and B represent linear regression lines with slopes significantly different from 0 (p values are shown).

Fig. 6.

Mean values for various parameters measured in the supernatant of canine RBCs stored for 35 days at different temperatures and the effect of washing, labeling, and concentrating these RBCs after 35-day storage on counts and hemoglobin levels. (IA-E) Supernatant mean ([notdef] SE) values for various parameters after 35-day storage of canine RBCs at different storage temperatures. RBCs stored at different temperatures are shown by different shades of black and gray vertical rectangular bars. Supernatant mean values after RBC are stored for 35 days for pH (IA), lactate (IB), NTBI (IC), TBI (ID), and log (CFH) (IE). (IIA) For the ^{51}Cr test sample the mean ([notdef] SE) plasma values for hemoglobin levels (top panels) and (IIB) RBC counts (bottom panels) at baseline (left panels) and after 35-day storage and

washing, ^{51}Cr labeling, and concentrating (right panels). CFH = cell-free hemoglobin; NTBI = non-transferrin-bound iron; TBI = transferrin-bound iron.

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